CellPress

Chemistry & Biology
Review

Chemical Biology Methods for Investigating G Protein-Coupled Receptor Signaling

Thomas Huber[1,*] and Thomas P. Sakmar[1,2,*]
[1]Laboratory of Chemical Biology & Signal Transduction, The Rockefeller University, New York, NY 10065, USA
[2]Department of Neurobiology, Care Sciences and Society, Division for Neurogeriatrics, Center for Alzheimer Research, Karolinska Institutet, 141 57 Huddinge, Sweden
*Correspondence: hubert@rockefeller.edu (T.H.), sakmar@rockefeller.edu (T.P.S.)
http://dx.doi.org/10.1016/j.chembiol.2014.08.009

G protein-coupled receptors (GPCRs) are targets for a quarter of prescription drugs. Despite recent progress in structural biology of GPCRs, only few key conformational states in the signal transduction process have been elucidated. Agonist ligands frequently display functional selectivity where activated receptors are biased to either G protein- or arrestin-mediated downstream signaling pathways. Selective manipulation of individual steps in the GPCR activation scheme requires precise information about the kinetics of ligand binding and the dynamics of downstream signaling. One approach is to obtain time-resolved information using receptors tagged with fluorescent or structural probes. Recent advances allow for site-specific introduction of genetically encoded unnatural amino acids into expressed GPCRs. We describe how bioorthogonal functional groups on GPCRs enable the mapping of receptor-ligand interactions and how bioorthogonal chemical reactions can be used to introduce fluorescent labels for single-molecule fluorescence applications to study the kinetics and conformational dynamics of GPCR signaling complexes ("signalosomes").

Seven-transmembrane helical G protein-coupled receptors (GPCRs) are highly dynamic membrane proteins that transmit extracellular signals across the membrane to activate and mediate several signaling pathways (Huber et al., 2008; Menon et al., 2001). GPCRs play fundamental roles in a wide spectrum of physiological and pathophysiological processes. In the classical paradigm of GPCR signaling, an extracellular ligand triggers ligand-induced conformational changes (Ye et al., 2009, 2010) in the receptor that leads to recruitment of cytosolic heterotrimeric G proteins, which in turn activate enzyme effectors to amplify the signal by generating a large number of cellular second messengers (Katritch and Abagyan, 2011; Steyaert and Kobilka, 2011). Recent advances in crystallography have provided high-resolution structures of a growing number of GPCRs in multiple conformational states, including a G protein-bound complex (Hollenstein et al., 2014; Kobilka and Schertler, 2008; Rasmussen et al., 2011), but there is now a pressing need for additional biophysical and biochemical techniques to achieve a precise understanding of the chemical basis and structural dynamics of receptor activation and allosteric mechanism.

One very active area of research involves the biology of GPCR oligomerization in cell membranes. In cell-based systems, GPCR oligomerization has been studied using resonance energy transfer (RET) assays that measure the proximity of donor and acceptor probes (fluorescent and/or bioluminescent) attached to target receptors (Ciruela et al., 2010). Although RET assays have provided extensive data concerning GPCR oligomerization (Pfleger and Eidne, 2005) and ligand-dependent receptor coupling to cellular proteins, there are a number of inherent limitations in these approaches (Bouvier et al., 2007; James et al., 2006; Salahpour and Masri, 2007). Single-molecule detection (SMD) fluorescence is another approach to visualize membrane protein oligomers in membrane bilayers (Calebiro et al., 2013; Hern et al., 2010; Kasai et al., 2011; Lin et al., 2014). In principle, single molecule tracking can detect the movement of individual oligomers and monitor association and dissociation events in real time. The recent applications of single-molecule tracking to study GPCRs were transformative because they provided evidence for the existence of oligomers in living cells at essentially physiological expression levels (Calebiro et al., 2013; Hern et al., 2010; Prummer et al., 2006; Snaar-Jagalska et al., 2013; Suzuki et al., 2005). However, the precise functional role of oligomerization, the structural basis for receptor association, and the contributions of membrane lipids to the process remain ill defined in part because of a lack of flexible and robust methods to tag receptors in biological membranes.

Fluorescence techniques are indispensable for monitoring the conformational states of purified receptors (Bockenhauer et al., 2011; Dunham and Farrens, 1999; Gether et al., 1997; Peleg et al., 2001) and receptors in live cells (Calebiro et al., 2013; Hern et al., 2010; Kasai et al., 2011; Kusumi et al., 2010; Lohse et al., 2012; Maurel et al., 2008; Vilardaga et al., 2009). In studies of enzyme catalysis, multicolor SMD fluorescence techniques allow the correlation of conformational dynamics of doubly labeled enzymes to the binding of their labeled substrates (Tinnefeld and Sauer, 2005). SMD approaches can in principle detect both the function and stoichiometry of discrete bimolecular complexes (Ha, 2013).

Transmembrane signaling is an allosteric process because the conformational change induced in the receptor upon ligand binding must be transmitted to the binding site of a cellular transducer protein, such as a G protein or β arrestin, on the opposite side of the membrane (Christopoulos and Kenakin, 2002). Different agonist ligands for the same receptor might be biased for a particular signaling pathway, leading to the

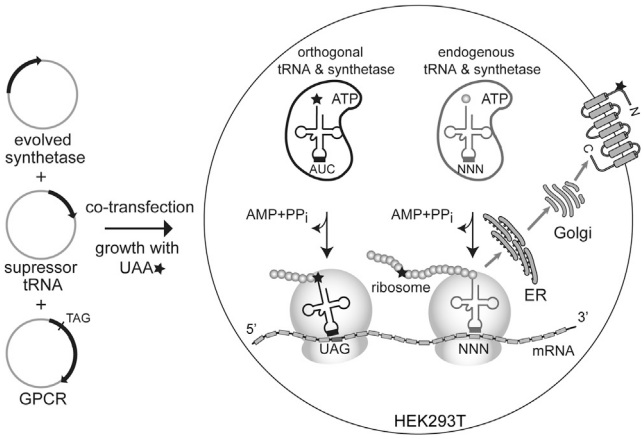

Figure 1. uaa Mutagenesis of GPCRs in Mammalian Cells
Amber stop codon (UAG) suppression technology is based on the presence of an orthogonal suppressor amino acyl-tRNA. The suppressor tRNA is charged with the uaa in a reaction catalyzed by an engineered orthogonal aminoacyl-tRNA synthetase. Heterologous expression of GPCRs in mammalian cells provides the most reliable approach to guarantee nativelike posttranslational modifications and function (Sarramegna et al., 2003). HEK293T or HEK293F cells are cotransfected with three different plasmids encoding the evolved synthetase gene, the suppressor tRNA gene, and the gene encoding the GPCR with an in-frame TAG mutation at a desired position. The orthogonal tRNA/synthetase pair from bacteria does not cross-react with endogenous mammalian tRNAs and synthetases, but the ribosome is able to utilize the charged tRNA. Culturing the transfected cells in the presence of the uaa results in protein translation, with suppression of the amber stop codon and site-specific incorporation of the uaa at a desired position to give a full-length protein (adapted from Daggett and Sakmar, 2011).

pharmacological concept of "functional selectivity" (Kenakin and Christopoulos, 2013). A number of models have been proposed to account for ligand bias and functional selectivity. One hypothesis is that biased signaling can be described by multiple signaling-competent receptor conformations that engage in alternative ternary complexes (Rajagopal et al., 2010). An alternative hypothesis is that agonist bias may be predicted by differential conformational changes in the ligand-receptor complex alone (Horst et al., 2013; Liu et al., 2012), but it is generally accepted that the fully active conformation is normally not reached without a G protein- or β arrestin-binding partner (Nygaard et al., 2013). Elucidating the molecular mechanism of receptor allosterism and signaling bias requires quantification of the strength of allosteric coupling. This strength is accessible due to the reciprocity principle (Fenton, 2008); i.e., the changes of agonist affinity due to binding of a transducer are equal in magnitude to the changes of the transducer affinity shift due to binding of the agonist. For example, the G protein-dependent shift in agonist affinity correlates with intrinsic agonist activity (Kent et al., 1980), and a similar correlation is found for β arrestin-dependent agonist affinity shifts (Gurevich et al., 1997). Some allosteric interactions exhibit neutral cooperativity (i.e., no observable shift in affinities), but they still show pronounced changes in ligand binding and dissociation kinetics (Christopoulos and Kenakin, 2002). Therefore, it has been argued that experiments to measure ligand dissociation kinetics are superior to study allosteric effects (Avlani et al., 2004; Kostenis and Mohr, 1996; Lazareno and Birdsall, 1995). We suggest that single-molecule fluorescence techniques facilitated by site-directed fluorescence labeling of GPCRs will be extremely useful to monitor the effects of G protein and β arrestin on ligand binding and dissociation kinetics.

In this review, we describe a transdisciplinary technology platform that addresses the challenges inherent in single-molecule studies of GPCRs. Our focus is on the chemical biology approaches that should enable future quantitative studies of GPCRs in biological membranes.

Amber Codon Suppression in Expressed GPCRs

Fluorescent reporters suitable for SMD fluorescence studies include fluorescent proteins (GFP/RFP), semiconductor nanocrystals (quantum dots), and small organic fluorophores (rhodamine or indocyanine dyes). Site-specific maleimide and methanethiosulfonate Cys chemistries have been used to attach conformation-sensitive probes, like spin labels in case of rhodopsin (Altenbach et al., 2008; Hubbell et al., 2003; Huber and Sakmar, 2008), or fluorescence labels for the β2-adrenergic receptor (Ghanouni et al., 2001). However, to achieve single-site labeling, this strategy ideally requires the introduction of a Cys residue by site-directed mutagenesis into a highly engineered "Cys-free" receptor. Since GPCRs contain multiple reactive Cys residues, to engineer a satisfactory receptor substrate is not trivial, especially since it is difficult to know a priori which Cys residues are important for function and how mutagenesis might alter receptor properties (Cordomí et al., 2013; Gether et al., 1997; Karnik et al., 1988). Even in cases where "Cys-free" receptors can be prepared and isolated, sulfhydryl chemistry is not bioorthogonal, and labeling receptors in a cellular context or in crude extracts is not feasible. Therefore, purification of a target receptor is required before thiol derivatization can be attempted.

To avoid targeting Cys residues, one strategy is to use unnatural amino acid (uaa) mutagenesis for site-specific introduction of unique functional groups that are not found in native proteins (Figure 1). Bioorthogonal chemical reactions can then be used to further derivatize the protein with fluorophores and other biophysical probes.

Twenty-five years ago, amber codon suppression (Goodman et al., 1968; Laski et al., 1982) was combined with chemical misacylation of tRNA (Hecht et al., 1978; Heckler et al., 1984) and in vitro protein translation as a new technology for site-specific incorporation of uaas with functional groups different from the 20 "proteinogenic" amino acids (Noren et al., 1989).

Stop codons (UAG, amber; UGA, opal; UAA, ochre) can be used to expand the genetic code since they are uniquely identified by the eukaryotic release factor 1 (eRF1) (Frolova et al., 1994; Song et al., 2000), whereas other codons typically are recognized each by multiple tRNA genes (Geslain and Pan, 2010; Lander et al., 2001; Lavner and Kotlar, 2005; Novoa et al., 2012). The overall efficiency of stop codon suppressor tRNAs is determined by competition with eRF1/eRF3. Amber stop codons are the least affected by eRF1/eRF3 competition (Gubbens et al., 2010), which makes the amber stop codon attractive for genetic encoding of uaas. Moreover, the frequency of the amber stop codon in the complete protein coding genes (CDSs) in *Homo sapiens* is only 23.5%, whereas ochre and opal are used at 29.4% and 47.1%, respectively (see http://www.kazusa.or.jp/codon) (Nakamura et al., 2000). Interestingly, some eukaryotic organisms do not use all three stop codons, but

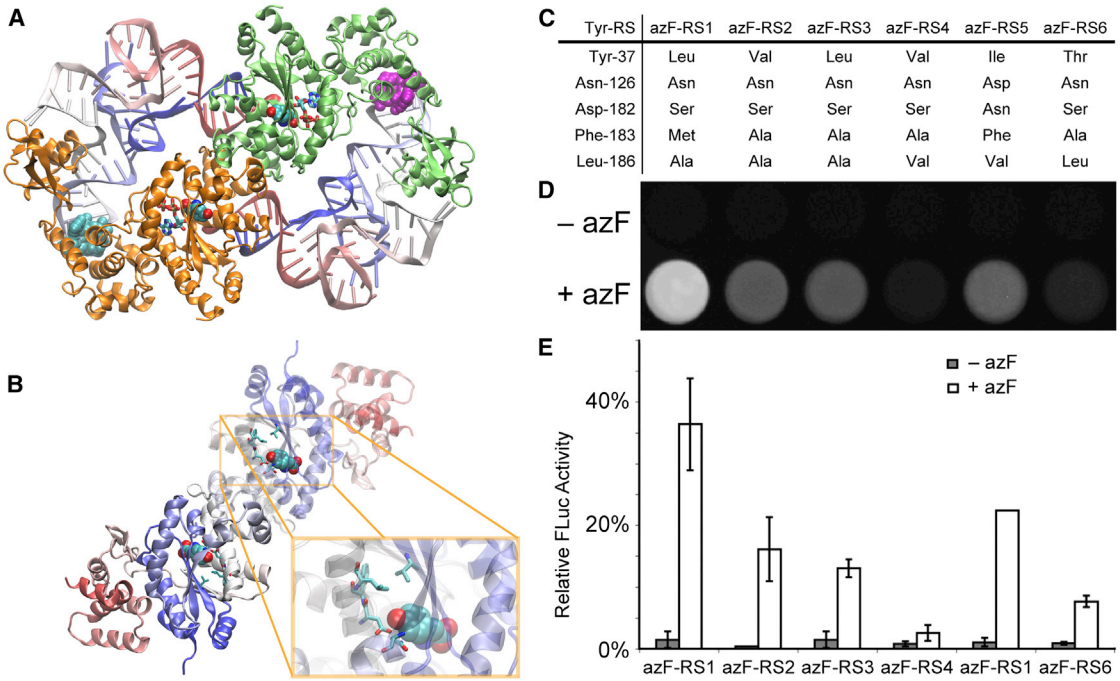

Figure 2. Engineering of Amino Acyl-tRNA Synthetases to Recognize uaa

(A) Crystal structure of *T. thermophilus* Tyr-tRNA synthetase (Tyr-RS) (Yaremchuk et al., 2002) shows class II mode of tRNA recognition in a class I synthetase (Ibba and Soll, 2000).

(B) Crystal structure of *E. coli* Tyr-RS dimer (Kobayashi et al., 2005) with closeup view of Tyr binding site residues engineered to recognize *para*-substituted Phe analogs while rejecting Tyr (Chin et al., 2003).

(C–E) Efficiency and specificity of mutant *E. coli* Tyr-RS variants recognizing *p*-azido-Phe (azF). Amber suppression efficiency in the human HEK293T cell line was evaluated using a luciferase reporter assay, which demonstrated efficient and specific incorporation of azF (Ye et al., 2008, 2009). Error bars represent the standard error of the mean of three replicate experiments.

use UGA as a stop codon and UAA/UAG as codons for Gln. Others use UAA/UAG as stop codons and UGA for Cys (Conard et al., 2012). The unusual amino acid pyrrolysine (Pyl/O) is incorporated in response to the UAG amber codon in certain methanogen archaebacteria (Srinivasan et al., 2002). Downregulation of eRF1 has been used to increase the efficiency of uaam (Carnes et al., 2003; Ilegems et al., 2004). The efficiency of translation termination is cell-type dependent; for example, HEK293 cells exhibit about 2% natural amber suppression, which is 10-fold less than Chinese hamster ovary cells (Ilegems et al., 2004). The natural amber suppression in mammalian cells is codon context sensitive (Cassan and Rousset, 2001), which theoretically should affect the efficiency of incorporation of uaas using the amber codon-suppression strategy. Several cytoplasmic tRNAs have been implicated in natural amber suppression in mammalian cells, and it is likely that Gln (and possibly Leu) residues will be incorporated as a result (Atkins and Gesteland, 2002; Beier and Grimm, 2001). Moreover, aminoglycoside antibiotics lead to substantial (up to 10%) read-through/suppression of amber stop codons in mammalian cells (Burke and Mogg, 1985; Floquet et al., 2012; Malik et al., 2010; Martin et al., 1989; Phillips-Jones et al., 1995).

A method utilizing an engineered archaebacterial tyrosyl-tRNA/synthetase pair in *E. coli* was reported where the archaeal pair was orthogonal to eubacterial tRNAs and synthetases, but the acylated tRNA is efficiently utilized by the *E. coli* ribosome. The CUA anticodon was introduced in the tRNA to act as an amber suppressor tRNA. The mutation of the synthetase was guided by crystallographic data (Figure 2) so that the successfully engineered synthetase would recognize the tyrosine analog *O*-methyl-Tyr and reject Tyr as substrate (Wang et al., 2001).

A similar strategy was used to introduce 3-iodo-Tyr by amber codon suppression in mammalian cells. The orthogonal pair tRNA/synthetase pair was a combination of *E. coli* Tyr-RS and *B. stearothermophilus* suppressor $tRNA^{Tyr}$ (Sakamoto et al., 2002). Later, the method was expanded to yeast (*S. cerevisiae*) using the *E. coli* tyrosyl-tRNA/synthetase pair orthogonal to the eukaryotic tRNAs and synthetases. The yeast system enabled extensive genetic selection to specifically and efficiently incorporate five different uaas (*p*-acetyl-, *p*-benzoyl-, *p*-azido-, *p*-methoxy-, and *p*-iodo-Phe) (Chin et al., 2003).

We built on these findings and optimized the methodology to improve dramatically the amber suppression efficiency by generating a tRNA expression plasmid carrying a chimeric gene of human and *B. stearothermophilus* suppressor $tRNA^{Tyr}$ (Ye et al., 2008). Our chimeric tRNA gene expressed well, formed an orthogonal pair with the *E. coli* Tyr-RS variants, and was efficiently processed and acylated with Tyr and analogs in the human HEK293T cell line (Ye et al., 2008). The biosynthesis of tRNA from our chimeric tRNA gene can be rationalized based on detailed studies of the splicing and processing of the human $tRNA^{Tyr}$ gene (MacPherson and Roy, 1986), which indicate that spicing of the primary transcript precedes processing to trim

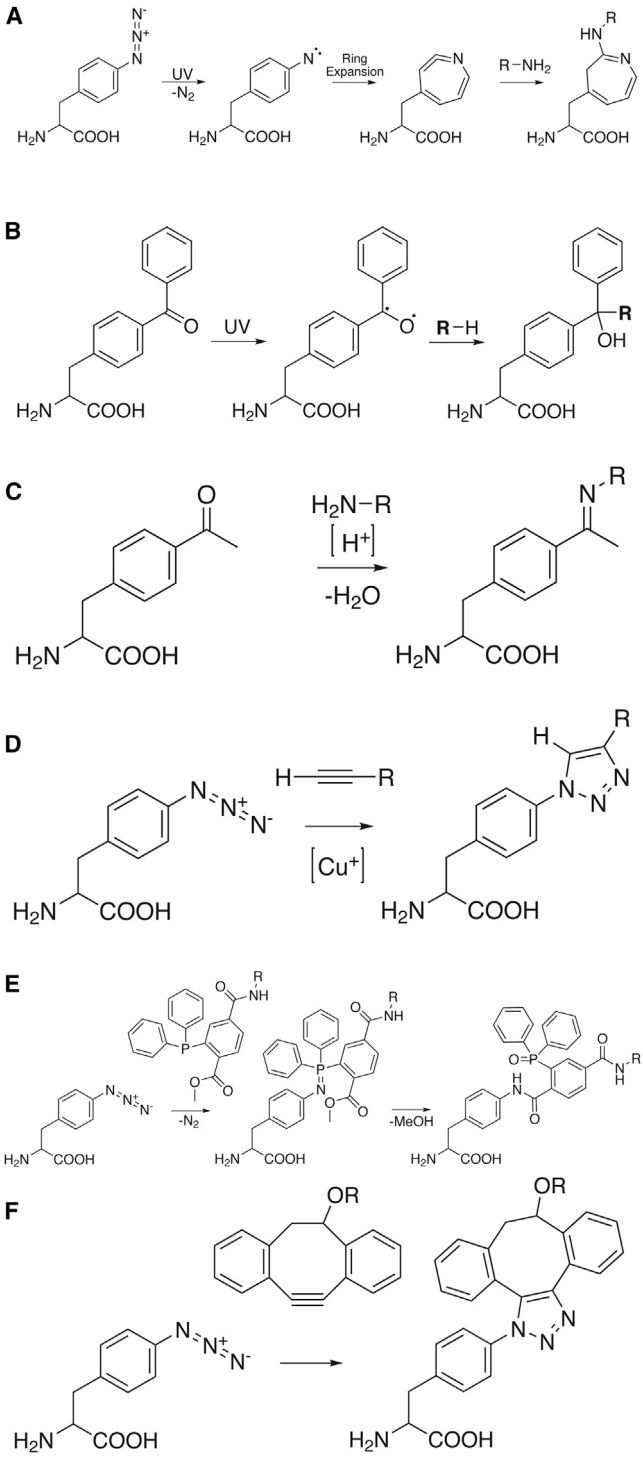

Figure 3. Photocrosslinking and Bioorthogonal Labeling Reactions
(A) Photocrosslinking reaction with *p*-azido-Phe (azF).
(B) Photocrosslinking reaction with *p*-benzoyl-Phe (BzF).
(C) Oxime and hydrazone ligation reactions of *p*-acetyl-Phe (AcF).
(D) CuAAC of azF with a terminal alkyne reagent results in a 1,2,3-triazole conjugate.
(E) Staudinger-Bertozzi ligation of azF with a phosphine reagent.
(F) SpAAC of azF with a DIBO reagent resulting in a 1,2,3-triazole conjugate.

the 5′ and 3′ ends of the pre-tRNA and attachment of the CCA sequence on the 3′ end (van Tol et al., 1987).

Besides bacteria (*E. coli*), yeast (*S. cerevisiae*), and mammalian cells in culture (Chin, 2014; Liu and Schultz, 2010), uaa mutagenesis methods have been developed for cultured insect cells (*Drosophila melanogaster* Schneider 2 cells) (Mukai et al., 2010), amphibian oocytes (*Xenopus laevis*) (Ye et al., 2013), and two animals, the nematode *Caenorhabditis elegans* (Greiss and Chin, 2011) and the arthropod *Drosophila melanogaster* (Bianco et al., 2012).

Genetically Encoded uaas to Facilitate Biophysical and Photochemical Crosslinking Studies of GPCRs

Using the strategy described above, we have been able to incorporate uaas such as *p*-benzoyl-L-phenylalanine (BzF), *p*-acetyl-L-phenylalanine (AcF), and *p*-azido-L-phenylalanine (azF) into heterologously expressed GPCRs in mammalian cells (Grunbeck et al., 2011, 2012; Huber et al., 2013; Naganathan et al., 2013a, 2013b; Ray-Saha et al., 2014; Tian et al., 2013, 2014; Valentin-Hansen et al., 2014; Ye et al., 2008, 2009).

The photolabile uaas, BzF and azF, enabled the mapping of ligand-binding sites on chemokine receptors (CXCR4 and CCR5) (Grunbeck et al., 2011, 2012; Ray-Saha et al., 2014) and on the substance P receptor NK1 (Valentin-Hansen et al., 2014). Photochemical crosslinking experiments were carried out in live cells prior to identification of crosslinked products. When crosslinks occur, the site of the linkage on the receptor is known since the photoreactive amino acid was genetically encoded at a specific known site in the primary structure. Crystal structure data or homology models were used to interpret quantitative crosslinking data. Cell-based signaling assays were carried out on engineered receptors in the same cells and under the same conditions used for subsequent crosslinking reactions. The "targeted photocrosslinking" method was also used to map monoclonal antibody (mAb) epitopes by crosslinking GPCR-mAb complexes (Ray-Saha et al., 2014).

UV illumination of aromatic azides, such as azF, forms the highly reactive nitrone that undergoes ring expansion and nucleophile addition, resulting in chemical crosslinks (Bräse et al., 2005) (Figure 3A). The long-wavelength UV light (365 nm) excites the benzophenone group in BzF to a diradicaloid triplet state, which either relaxes back to the benzophenone ground state or reacts with surrounding molecular groups by H abstraction and recombination to a photocrosslinked product (Dormán and Prestwich, 1994) (Figure 3B). A chemical crosslinking method was also reported using a *p*-fluoroacetyl-Phe (Ffact) residue in the receptor that reacts with a nearby Cys residue introduced in a peptide ligand (Coin et al., 2013).

To probe the biology of ion channels, electrophysiology experiments with ionotropic glutamate receptors (iGluR) in *Xenopus* oocytes (Zhu et al., 2014) and HEK293 cells (Klippenstein et al., 2014) have demonstrated that the system for azF and BzF incorporation (Ye et al., 2008, 2009) enables photocrosslinking-based conformational switching of AMPA- and NMDA-type iGluRs.

To study conformational dynamics of rhodopsin photoactivation, the unique infrared vibrational spectrum of the azido group was probed in a series of site-specific azF mutants.

Fourier-transform infrared (FTIR)-difference spectra were measured to show that the electrostatic environments of some residues change very early in the activation process (Ye et al., 2009, 2010). These types of receptor dynamics experiments can be correlated with high-resolution structures of receptor activation intermediates to gain significant insights into the biology of receptor signaling.

Bioorthogonal Labeling Strategies

Different bioorthogonal labeling strategies using AcF or azF have been compared (Huber et al., 2013; Naganathan et al., 2013a, 2013b; Tian et al., 2013, 2014; Ye et al., 2008). Ketone groups incorporated into a variety of proteins and other macromolecules have facilitated novel ligation strategies such as hydrazone and oxime ligation reactions that utilize aminooxy/hydroxylamine (R = –O–R') or hydrazide (R = –NH–CO–R'') reagents (Figure 3C). Under approximately physiological conditions, ketones and aldehydes react specifically with hydrazide (or aminooxy) reagents through hydrazone (or oxime) ligation (Bayer et al., 1988; Wang et al., 2003). The observation that aminooxy derivatives have faster labeling kinetics at close to neutral pH as compared with hydrazides is advantageous for protein labeling. These reactions have been used to label proteins at genetically encoded N-terminal aldehyde tags (Shi et al., 2012), enzymatically coupled chemical handles (Chen et al., 2005), engineered cell surface oligosaccharides (Hang and Bertozzi, 2001; Mahal et al., 1997), and with more flexibility at site-specifically incorporated uaas (Cornish et al., 1996; de Graaf et al., 2009; Fleissner et al., 2009; Wang and Schultz, 2004; Wang et al., 2003; Ye et al., 2008; Zhang et al., 2003).

We observed substantial covalent modification of wild-type rhodopsin and other control proteins that did not contain AcF (Huber et al., 2013; Tian et al., 2014; Ye et al., 2008). This undesired reactivity was caused by cellular oxidation processes that convert some amino acid residues (Arg, Cys, His, Lys, and Pro) to carbonyl derivatives that are reactive toward hydrazide and aminooxy reagents (Ahn et al., 1987; Stadtman and Levine, 2003). This ''protein carbonylation'' phenomenon severely limits the applicability of ketone-selective bioorthogonal labeling reactions (Grimsrud et al., 2008; Stadtman, 1993). The degree of target-specific background labeling due to protein carbonylation will be roughly proportional to the protein's molecular mass, with about one reactive carbonyl per each thousand amino acid residues (Huber et al., 2013; Ye et al., 2008).

Advantages of Bioorthogonal Azido Tags

The azido group has been reported to be a superior handle for bioconjugation reactions (Bräse et al., 2005; Debets et al., 2010b; Schilling et al., 2011; van Berkel et al., 2011). Azide provides an alternative bioorthogonal handle that can be incorporated in proteins by uaa mutagenesis. Azides react specifically with phosphines through the Staudinger-Bertozzi ligation (see below) (Kiick et al., 2002; Saxon and Bertozzi, 2000), with alkyne reagents through Cu(I)-catalyzed [3+2] azide-alkyne cycloaddition (CuAAC) (Meldal and Tornøe, 2008; Rostovtsev et al., 2002; Tornøe et al., 2002), with oxanorbornadienes through tandem [3+2] cycloaddition-retro-Diels-Alder (tandem crDA) (van Berkel et al., 2007), or with cyclooctynes through strain-promoted azide-alkyne cycloaddition (SpAAC) (Agard et al., 2004; Debets et al., 2011; Link et al., 2006; Sletten and Bertozzi, 2011).

The discovery of a cell-compatible and selective ligation reaction modeled after the classical Staudinger reaction between an azide and a phosphine inspired the nascent field of bioorthogonal chemical reactions. This reaction, sometimes called Staudinger-Bertozzi ligation, is a highly selective reaction between modified triarylphosphines and azides to form an aza-ylide intermediate that undergoes cyclization followed by hydrolysis to form a stable amide-linked adduct (Köhn and Breinbauer, 2004; Saxon and Bertozzi, 2000; Saxon et al., 2002; Tsao et al., 2005). It has been used to modify cell surface glycans with haptenes, epitope tags, and fluorescent probes (Chang et al., 2007; Cohen et al., 2010; Hangauer and Bertozzi, 2008; Kiick et al., 2002; Laughlin and Bertozzi, 2007; Lemieux et al., 2003; Saxon and Bertozzi, 2000). It has also been used to label azido amino acids in proteins in residue-specific (Kiick et al., 2002) or site-specific (Mukai et al., 2010; Tsao et al., 2005) manners. In another study, FLAG phosphine (FLAG is a peptide epitope for a useful well-characterized mAb) was used to label azido-bearing biotin. The FLAG biotin was then used to tag an endogenous biotin acceptor protein (Slavoff et al., 2008).

Despite the susceptibility of phosphines used for the Staudinger ligation to air oxidation (Agard et al., 2004) and their substoichiometric modification of azF in recombinant proteins (Huber et al., 2013; Yanagisawa et al., 2008) and azido sugars (Agard et al., 2006), their compatibility to cell-based applications (Prescher et al., 2004) makes them attractive candidates for further study.

In studies on GPCR labeling, we found that the reaction yields essentially background-free product, consistent with a truly bioorthogonal reaction (Huber et al., 2013). However, despite reports of stoichiometric conjugation of azF in other recombinant proteins with fluorescein-phosphine (Tsao et al., 2005), we found that substoichiometric conjugation to azF rhodopsin is not the result of its relatively slow reaction kinetics, but possibly due to a classical Staudinger reaction instead of the desired Staudinger-Bertozzi ligation. Consistent with our findings, others have reported incomplete modification of azF and a longer azido-containing pyrrolysine analog by Staudinger ligation, which was attributed to the shorter linker length between the backbone and the azide (Yanagisawa et al., 2008).

In order to advance biological applications of novel labeling methods, we developed a strategy to detect modified receptors in live cells. We used the Staudinger ligation to modify azF residues in the chemokine receptor CCR5 with a FLAG peptide-epitope tag (Figure 4). One application of this technique is a semihigh-throughput method to identify sites accessible for site-specific labeling with other reagents like fluorophores. Accessible sites are then used in a preparative method to obtain purified receptors for SMD fluorescence work (Naganathan et al., 2013b). In contrast to alternative methods based on labeling Cys residues, such as the substituted Cys accessibility method (Akabas et al., 1992; Zhu and Casey, 2007), the new method does not display cross-reactivity with other Cys residues that are present in high abundance in the cell.

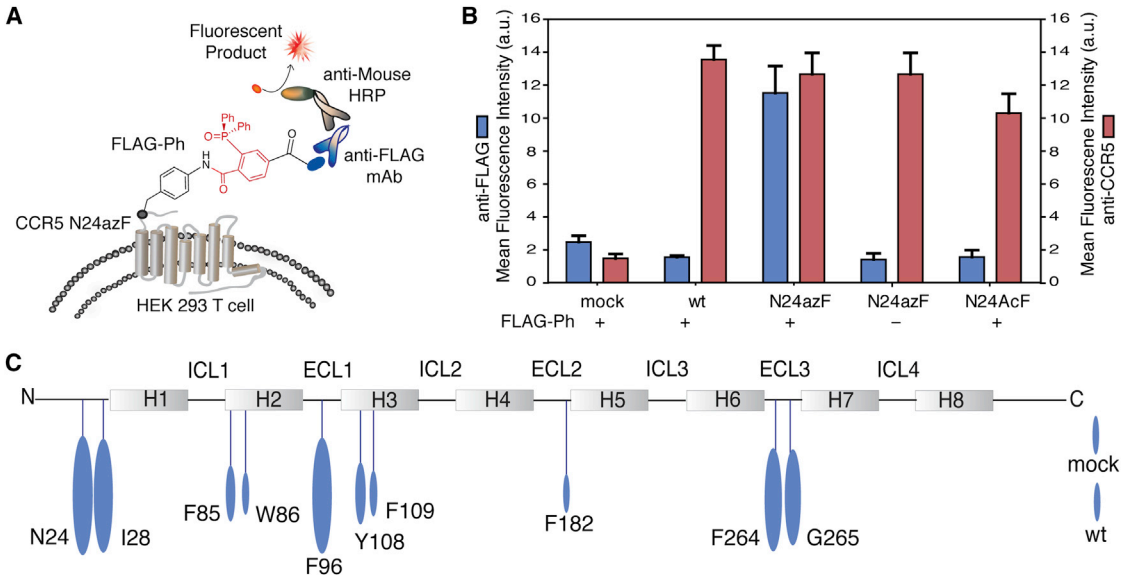

Figure 4. Staudinger-Bertozzi Ligation to Label azF Residues in CCR5

(A) Cell-based ELISA strategy to detect low abundance GPCRs labeled with FLAG-phosphine in a live cell context.

(B) Control experiments with wild-type CCR5, CCR5 with the keto amino acid AcF, and CCR5 with the azido amino acid azF demonstrate that specific labeling of exposed resides is only achieved with azF-containing receptors. Error bars represent the standard error of the mean of three or more replicate experiments.

(C) Accessibility scan of azF residues incorporated in different position in CCR5 indicates that only a fraction of residues in a folded receptor is accessible to the reactive label (Naganathan et al., 2013b).

Bioorthogonal Cycloaddition Reactions

The catalytic action of Cu(I) in the Huisgen 1,3-dipolar azide-alkyne cycloaddition (Huisgen et al., 1967) was reported by two groups (Rostovtsev et al., 2002; Tornøe et al., 2002). The reaction is now commonly called copper-catalyzed [3+2] azide-alkyne cycloaddition (CuAAC) (Figure 3D). A significant disadvantage for protein applications is that the reaction may damage Cys, Met, and His residues in proteins and side reactions can form reactive aldehydes that result in undesired protein adducts, crosslinks, and precipitation. Another problem is that Cu(I) has extremely high affinity for Cys residues (Rubino et al., 2011; Wu et al., 2010), and complete removal of the catalyst is a challenge in protein conjugation reactions. Despite these problems, the CuAAC reaction is very attractive since the terminal alkyne and azide groups each are very small and the resulting triazole linker is rather compact. The CuAAC reaction has all the qualities of a "Click" reaction (Kolb et al., 2001); it is orthogonal to other common reactions and functional groups (Wong and Zimmerman, 2013), and it has made an enormous impact in chemistry (Meldal and Tornøe, 2008), including drug discovery (Devigny et al., 2011; Sakmar, 2011; Thirumurugan et al., 2013).

In the search for alternatives to CuAAC, reactions of azides with cyclooctyne, a ring-strained alkyne, were evaluated (Agard et al., 2004). This reaction is also known as the Wittig reaction, as Wittig and Krebs identified the product, 1-phenyl-4.5-cyclooctenо-1.2.3-triazol (Wittig and Krebs, 1961), of an explosive reaction between cyclooctyne and phenyl azide, described several years earlier (Blomquist and Liu, 1953). The term strain-promoted [3+2] azide-alkyne cycloaddition (SpAAC) was coined for this new bioorthogonal bioconjugation reaction, which had superior biocompatibility as compared with CuAAC and higher reaction efficiency as compared with the Staudinger-Bertozzi reaction (Agard et al., 2006).

Dibenzocyclooctyne (DIBO) derivatives were developed for SpAAC (Ning et al., 2008) based on a report of the spontaneous reaction of phenyl azide with DIBO (Seitz et al., 1969), aza-dibenzocyclooctynes were synthesized (DIBAC or DBCO) (Debets et al., 2010a), biarylazacyclooctynones (BARACs) were introduced (Jewett et al., 2010), and efficient syntheses of much more hydrophilic bicyclononynes (BCNs) was demonstrated (Dommerholt et al., 2010).

The Background Problem in SpAAC Reactions

Although SpAAC reactions involving cyclooctynes were thought to be biocompatible (Prescher and Bertozzi, 2005) and specific (Sletten and Bertozzi, 2011), the degradation of one cyclooctyne (BARAC) in the presence of thiols (glutathione) has been reported (Jewett et al., 2010), and background reactions with Cys residues in proteins have been attributed to a broad spectrum of cyclooctynes. Therefore, SpAAC is not strictly bioorthogonal due to the reaction of cyclooctynes with Cys residues in a thiol-yne addition reaction (van Geel et al., 2012). However, we demonstrated the general utility of the SpAAC reaction targeting azF residues with DIBO reagents in GPCRs and concluded that it is a satisfactory choice for labeling expressed receptors with fluorophores and other probes (Huber et al., 2013; Naganathan et al., 2013a; Tian et al., 2013, 2014).

The kinetics of the SpAAC reaction of DIBO labeled probes with azF residues in rhodopsin was up to 400-fold faster than similar reactions of model compounds in organic solution (Debets et al., 2011; Ning et al., 2008). This rate enhancement was dependent on the labeling position. A similar effect was

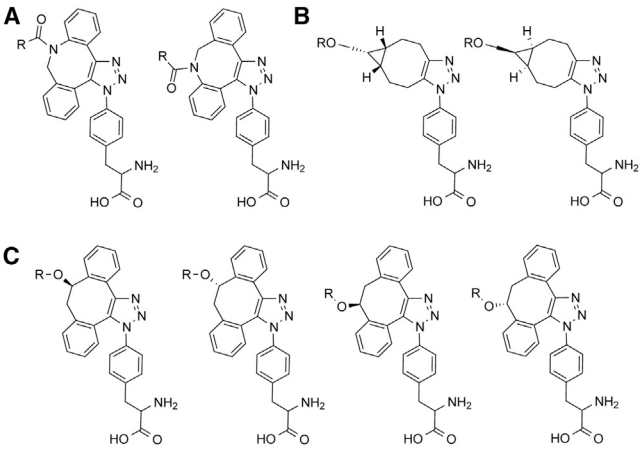

Figure 5. Regioisomeric and Stereoisomeric Forms of SpAAC Conjugates

(A) The reaction of DBCO and azF results in *anti*- and *syn*-regioisomers.

(B) Bicyclo[6.1.0]nonyne (BCN) derivatives exist in two forms, *endo* and *exo*. The reaction of *endo*-BCN with azF generates a new chiral center and two diastereomeric reaction products.

(C) DIBO conjugated dyes are typically racemic mixtures, and the chiral ring carbon of DIBO determines further enantiomeric forms of the triazole conjugate (S-*anti*, R-*anti*, S-*syn*, and R-*syn*).

described for a related cyclooctyne (DBCO) reaction in bacterially expressed GFP (Reddington et al., 2012). An increased local concentration of the cyclooctyne group is likely due to the lipophilic nature of DIBO (Debets et al., 2011). In fact, our results indicate efficient labeling of azF residues that are likely exposed to the hydrophobic core of the detergent micelles stabilizing the purified receptor preparations. Partitioning of the DIBO moiety into the micelles would lead to increased local concentration available for SpAAC (Tian et al., 2014).

Interestingly, the SpAAC reaction of Alexa488-DIBO with wild-type rhodopsin results in approximately a 1% azF-independent background labeling. This amount of background labeling is substantially less than the amount (10%∼30%) observed for AcF-independent background labeling of wild-type rhodopsin with fluorescein-hydrazide (Huber et al., 2013; Tian et al., 2014; Ye et al., 2008). Based on the amount of free Cys in rhodopsin (Blaskovic et al., 2013; Karnik et al., 1988; Mielke et al., 2002; Resek et al., 1993), we estimate that from two to eight Cys residues are potentially reactive toward DIBO. Consequently, a 1% background labeling of wild-type rhodopsin would correspond to a selectivity factor of DIBO for azF over Cys from 200:1 to 800:1, which is satisfactory bioorthogonality for a purified protein system.

While a small degree of background labeling is inconsequential for SMD fluorescence experiments with purified receptors, the abundance of Cys residues in cellular proteins severely limits the applicability of SpAAC for experiments in cells. Cys-mediated labeling of the proteome in the human HeLa cell line was reported (van Geel et al., 2012). One estimate counted 214,000 Cys residues encoded in the mammalian genome (Jones, 2010). Amino acid analysis of mammalian cells shows a Cys content of about 0.26% (Okayasu et al., 1997). The protein content of a single HeLa cell can be estimated to be 247 pg (Bosmann et al., 1968), which would correspond to 3 billion Cys residues per cell (in proteins). Considering our estimated selectivity factor of DIBO for azide over Cys, even in the best case scenario, at least a few million Cys residues per cell would be labeled as background. Even the best expressing GPCR rarely yields more than about 6 million copies per cell (Sarramegna et al., 2003), which is on the same order of magnitude as the expected Cys-based background labeling. The problem of Cys background is even more aggravated for receptor densities typically desired for SMD fluorescence tracking experiments (about 1 μm^{-2}) (Calebiro et al., 2013; Hern et al., 2010). Considering the surface area of the plasma membrane (Sommerhage et al., 2008), this density corresponds to only 2,500 receptors per cell. Clearly, additional methods to enhance specificity will be necessary to enable routine site-specific labeling and observation of low abundance proteins directly in cells.

In the meantime, labeled receptors can be used to study the pharmacology of ligand uptake and release reactions. For example, the SpAAC reaction was used to label site specifically and quantitatively azF-tagged rhodopsin with Alexa488-DIBO. The kinetics of ligand uptake and release in labeled receptor could be measured using fluorescence RET between the Alexa488 and the covalently bound ligand, 11-*cis*-retinal, in a membrane-mimetic bicelle system (Tian et al., 2014).

Progress toward Single-Molecule Studies of Bioorthogonally Labeled GPCRs

Currently, there are several commercially available cyclooctynes conjugated to fluorescence dyes suitable for SMD fluorescence experiments. DBCO is also known as DIBAC or ADIBO, depending on the substitution on the amide linker. DIBO conjugates appear to form less unwanted reactions with thiols as compared with BCN or DIBAC (van Geel et al., 2012). On the other hand, BCN is much less hydrophobic than DIBO or DIBAC (Debets et al., 2011), which are problematic in combination with hydrophobic dyes such as Atto647N (Yao et al., 2012). We anticipate that synthesis of fluorescent probes based on these hydrophilic cyclooctynes, optimized linkers and novel fluorophores with additional functionalities, such as triplet state quenchers (Altman et al., 2012), will facilitate practical application of SpAAC to label proteins for SMD fluorescence experiments.

Preparation of Alexa647-DIBO site specifically conjugated to azF-labeled rhodopsin was achieved by labeling of the detergent-solubilized, purified receptor immobilized on an immunoaffinity matrix (Tian et al., 2013). A model of the Alexa647-DIBO-azF-rhodopsin conjugate illustrates the linker length and the relative size of the fluorophore as compared with the receptor (Figures 5 and 6). One can imagine that the long and flexible linker generates uncertainty in the position of the fluorophore, which might be problematic for distance measurements using fluorescence RET. The RET efficiency depends on the distance between the donor and acceptor fluorophores and the orientational factor κ^2 that can assume values from zero to four (Förster, 1948; Stryer and Haugland, 1967). Interestingly, the intuitive solution to shorten the linker length to get a better definition of the dye position may in fact be counterproductive and increase the uncertainty in calculations of the distance from the transfer efficiency due to nonrandom orientations ($\kappa^2 \neq 2/3$) (Sindbert et al., 2011). Computational methods, such

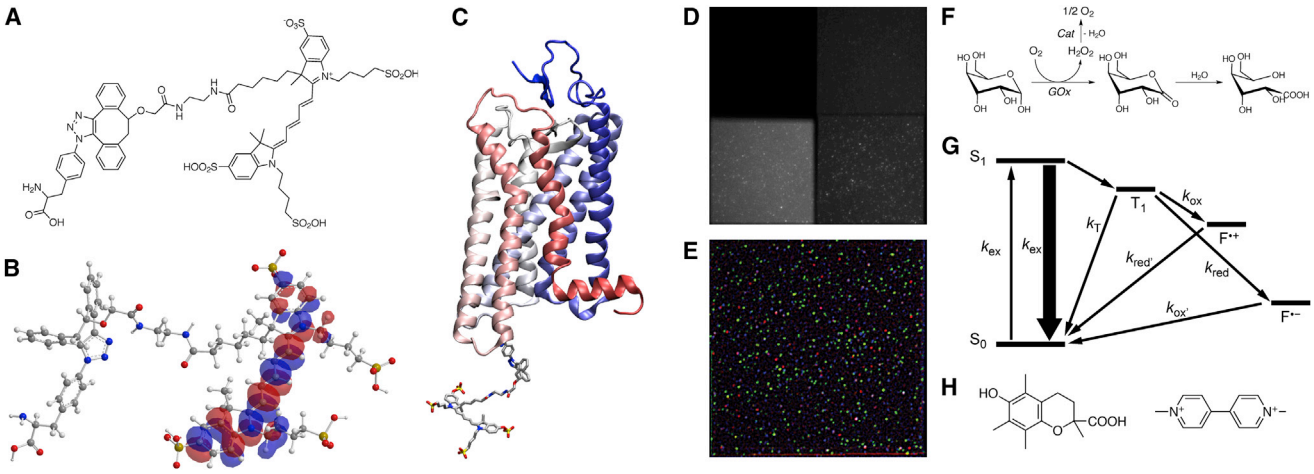

Figure 6. Three Color Single-Molecule Fluorescence Experiments with Alexa-DIBO Labeled azF-rhodopsin

(A) Alexa647 is a typical indocyanine-type fluorophore rendered hydrophilic by multiple sulfonate groups (White et al., 2006). The parent carboxylic acid form is linked via an ethylenediamine linker to carboxymethylated DIBO, shown as the *anti*-triazolyl conjugate with azF.

(B) A 3D conformation of the Alexa647-DIBO-azF conjugate generated by semiempirical quantum mechanics energy optimization. Note the relative orientation of two benzo groups relative to the triazole on the cyclooctene. A representative Hückel molecular orbital is shown to illustrate the conjugation of the indocyanine fluorophore.

(C) Alexa647-DIBO-azF manually modeled in position 144 of rhodopsin.

(D–H) Multicolor single-molecule detection total-internal reflection fluorescence imaging of a mixture of azF-rhodopsin molecules labeled with the rhodamine dye Alexa488-DIBO and the two cyanine dyes, Alexa555-DIBO and Alexa647-DIBO. (D) Raw image obtained with a QuadView image splitting device on an EM-CCD camera with single photon sensitivity with simultaneous 488, 561, and 642 nm laser excitation (Tian et al., 2013).

(E) Pseudocolor image computed from the raw image using elastic channel registration (Arganda-Carreras et al., 2006) and optimal filtering (Sage et al., 2005).

(F) Oxygen is reduced by an enzymatic oxygen scavenger system.

(G) The photophysics of a fluorescence dye may be described by a Jablonski diagram with the ground and first excited singlet states, S_0 and S_1, and the lowest triplet state, T_1. The long-lived T_1 state is quenched by molecular oxygen forming highly reactive singlet oxygen, but in presence of an oxygen scavenger, other methods are necessary to deplete the T_1 state, which otherwise is highly susceptible to photobleaching (Vogelsang et al., 2008).

(H) Trolox and methyl viologen form a reducing and oxidizing system that rapidly depletes the T_1 state.

as geometrical accessible volume simulations or molecular dynamics simulations of the position distribution of the dye and the linker dynamics can improve the accuracy of RET-based distance measurements with dyes on longer linkers (Best et al., 2007; Merchant et al., 2007; Muschielok et al., 2008; Schröder et al., 2005; Sindbert et al., 2011).

Strategies to Attach Multiple Labels

The full potential of uaa mutagenesis will be harnessed when it is combined with other protein modifications, such as protein fusion tags for enzymatic labeling tags (SNAP, CLIP, etc.) or autofluorescent proteins (e.g., GFP), classical Cys modification, or native protein ligation (Beck-Sickinger and Budisa, 2012). SNAP and CLIP are fast labeling tags with reactions rates 10^4–10^5 M^{-1} s^{-1} (Hinner and Johnsson, 2010), but these tags are 182-residue polypeptides (19.4 kDa) and are typically limited as fusion proteins to the N- or C-terminal parts of a target protein. In case of GPCRs, attaching a tag to the N-terminal tail of the receptor might result in loss of function. For example, the protease-activated receptor 1 (PAR1) is activated by a protease that cleaves its N-terminal segment (Ludeman et al., 2005). Another example is the adhesion GPCRs in which the N-terminal domain undergoes autoproteolytic cleavage or is swapped with other proteins (Araç et al., 2012).

Other polytopic membrane proteins, such as the multidrug resistance protein 1, have both N and C termini located on the cytoplasmic side (Bibi and Béjà, 1994), and protein N- or C-terminal fusion tags would not allow labeling of the extracellular surface. On the other hand, bioorthogonal-labeling reactions utilizing unique chemical handles on uaas enable labeling on virtually any surface-exposed site on an expressed protein. Besides the labeling strategies discussed above, other uaas based on engineered pyrrolysine-tRNA synthetase, such as a BCN derivative of pyrrolysine, enable Diels-Alder cycloaddition reactions with tetrazines (Lang et al., 2012) and SpAAC reactions with azido-containing labels (Borrmann et al., 2012).

In summary, the convergent technology platform described above provides a new approach to study the dynamics of GPCR signalosomes by SMD fluorescence methods. Tethered membrane "chip" systems can be used to study the kinetics of ligand-receptor interactions and receptor dynamics (Figure 7) while other bioorthogonal labeling methods hold promise in addressing the biology of GPCR oligomerization and functional selectivity in live-cell systems.

ACKNOWLEDGMENTS

We acknowledge the generous support of the Danica Foundation and the Crowley Family Foundation. Support for this project was provided by a grant from the Robertson Foundation. This work was also been generously supported by an International Research Alliance with Professor Thue W. Schwartz at The Novo Nordisk Foundation Center for Basic Metabolic Research (http://www.metabol.ku.dk) through an unconditional grant from the Novo Nordisk Foundation to University of Copenhagen. We also acknowledge and thank the following Sakmar Lab members who contributed to this work: Kelly Daggett, Amy Grunbeck, Manija Kazmi, Adam Knepp, Saranga Naganathan, Minyoung Park, Sarmistha Ray-Saha, Palavi Sachdev, He Tian, Louise Valentin-Hansen, and Shixin Ye.

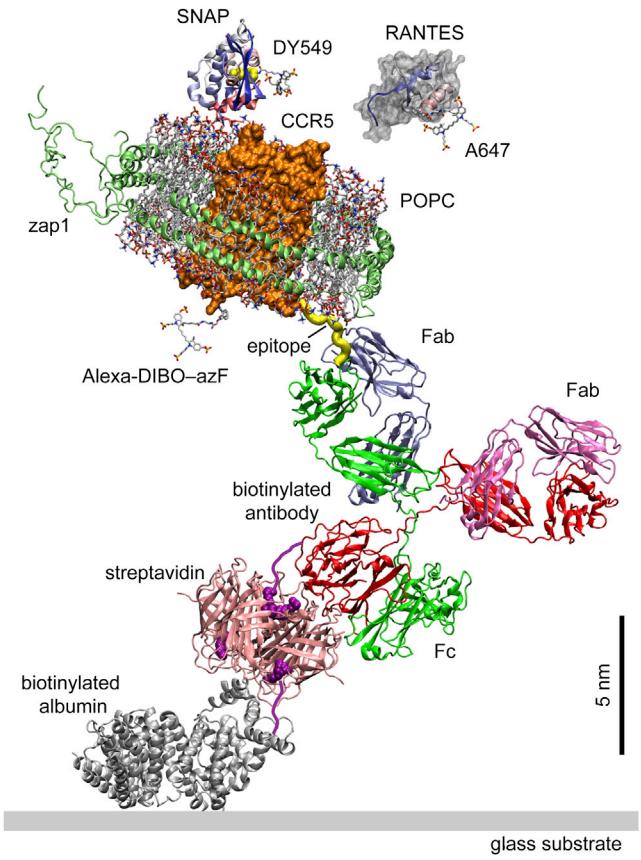

Figure 7. SMF Experiments with GPCR Signalosomes

The conceptual design of a supramolecular tethered assembly to study GPCRs by multicolor SMD-TIRF imaging. Crystal structures of representative proteins, albumin (Protein Data Bank [PDB]: 1GNJ), avidin (PDB: 1AVD), IgG1 (PDB: 1IGY), CCR5 (PDB: 4MSB), human O^6-alkylguanine-DNA alkyltransferase (AGT) (PDB: 1EH6), and AOP-RANTES (PDB: 1B3A), are manually arranged to illustrate how biotinylated albumin is physically adsorbed to the glass surface and is linked by (strept-) avidin to a biotinylated antibody. The antigen-binding fragment (Fab) of the antibody captured the receptor (CCR5) by an engineered C-terminal epitope. CCR5 is embedded in a nanoscale apolipoprotein-bound bilayer (Banerjee et al., 2008; Knepp et al., 2011). TIRF allows efficient excitation of fluorophores in the evanescent wave that extends beyond the glass surface into the aqueous solution (Axelrod et al., 1984). The receptor is labeled with an N-terminal engineered AGT tag (SNAP) (Keppler et al., 2003) and/or with SpAAC modified, genetically encoded azF residues (Alexa-DIBO-azF). Binding and release of fluorescent chemokines (RANTES-A647) can be detected in FRET or two-color single-molecule colocalization imaging experiments (Huber and Sakmar, 2011; Tian et al., 2013).

REFERENCES

Agard, N.J., Prescher, J.A., and Bertozzi, C.R. (2004). A strain-promoted [3 + 2] azide-alkyne cycloaddition for covalent modification of biomolecules in living systems. J. Am. Chem. Soc. *126*, 15046–15047.

Agard, N.J., Baskin, J.M., Prescher, J.A., Lo, A., and Bertozzi, C.R. (2006). A comparative study of bioorthogonal reactions with azides. ACS Chem. Biol. *1*, 644–648.

Ahn, B., Rhee, S.G., and Stadtman, E.R. (1987). Use of fluorescein hydrazide and fluorescein thiosemicarbazide reagents for the fluorometric determination of protein carbonyl groups and for the detection of oxidized protein on polyacrylamide gels. Anal. Biochem. *161*, 245–257.

Akabas, M.H., Stauffer, D.A., Xu, M., and Karlin, A. (1992). Acetylcholine receptor channel structure probed in cysteine-substitution mutants. Science *258*, 307–310.

Altenbach, C., Kusnetzow, A.K., Ernst, O.P., Hofmann, K.P., and Hubbell, W.L. (2008). High-resolution distance mapping in rhodopsin reveals the pattern of helix movement due to activation. Proc. Natl. Acad. Sci. USA *105*, 7439–7444.

Altman, R.B., Terry, D.S., Zhou, Z., Zheng, Q., Geggier, P., Kolster, R.A., Zhao, Y., Javitch, J.A., Warren, J.D., and Blanchard, S.C. (2012). Cyanine fluorophore derivatives with enhanced photostability. Nat. Methods *9*, 68–71.

Araç, D., Boucard, A.A., Bolliger, M.F., Nguyen, J., Soltis, S.M., Südhof, T.C., and Brunger, A.T. (2012). A novel evolutionarily conserved domain of cell-adhesion GPCRs mediates autoproteolysis. EMBO J. *31*, 1364–1378.

Arganda-Carreras, I., Sorzano, C.O.S., Marabini, R., Carazo, J.M., Ortiz-De-Solorzano, C., and Kybic, J. (2006). Consistent and elastic registration of histological sections using vector-spline regularization. CVAMIA: Computer vision approaches to medical image analysis. In Lecture Notes in Computer Science, *vol. 4241*, R.R. Beichel and M. Sonka, eds. (2006). (Berlin/Heidelberg: Springer), pp. 85–95.

Atkins, J.F., and Gesteland, R. (2002). Biochemistry. The 22nd amino acid. Science *296*, 1409–1410.

Avlani, V., May, L.T., Sexton, P.M., and Christopoulos, A. (2004). Application of a kinetic model to the apparently complex behavior of negative and positive allosteric modulators of muscarinic acetylcholine receptors. J. Pharmacol. Exp. Ther. *308*, 1062–1072.

Axelrod, D., Burghardt, T.P., and Thompson, N.L. (1984). Total internal reflection fluorescence. Annu. Rev. Biophys. Bioeng. *13*, 247–268.

Banerjee, S., Huber, T., and Sakmar, T.P. (2008). Rapid incorporation of functional rhodopsin into nanoscale apolipoprotein bound bilayer (NABB) particles. J. Mol. Biol. *377*, 1067–1081.

Bayer, E.A., Ben-Hur, H., and Wilchek, M. (1988). Biocytin hydrazide—a selective label for sialic acids, galactose, and other sugars in glycoconjugates using avidin-biotin technology. Anal. Biochem. *170*, 271–281.

Beck-Sickinger, A.G., and Budisa, N. (2012). Genetically encoded photocrosslinkers as molecular probes to study G-protein-coupled receptors (GPCRs). Angew. Chem. Int. Ed. Engl. *51*, 310–312.

Beier, H., and Grimm, M. (2001). Misreading of termination codons in eukaryotes by natural nonsense suppressor tRNAs. Nucleic Acids Res. *29*, 4767–4782.

Best, R.B., Merchant, K.A., Gopich, I.V., Schuler, B., Bax, A., and Eaton, W.A. (2007). Effect of flexibility and cis residues in single-molecule FRET studies of polyproline. Proc. Natl. Acad. Sci. USA *104*, 18964–18969.

Bianco, A., Townsley, F.M., Greiss, S., Lang, K., and Chin, J.W. (2012). Expanding the genetic code of Drosophila melanogaster. Nat. Chem. Biol. *8*, 748–750.

Bibi, E., and Béjà, O. (1994). Membrane topology of multidrug resistance protein expressed in Escherichia coli. N-terminal domain. J. Biol. Chem. *269*, 19910–19915.

Blaskovic, S., Blanc, M., and van der Goot, F.G. (2013). What does S-palmitoylation do to membrane proteins? FEBS J. *280*, 2766–2774.

Blomquist, A.T., and Liu, L.H. (1953). Many-membered carbon rings. VII. Cyclooctyne. J. Am. Chem. Soc. *75*, 2153–2154.

Bockenhauer, S., Fürstenberg, A., Yao, X.J., Kobilka, B.K., and Moerner, W.E. (2011). Conformational dynamics of single G protein-coupled receptors in solution. J. Phys. Chem. B *115*, 13328–13338.

Borrmann, A., Milles, S., Plass, T., Dommerholt, J., Verkade, J.M., Wiessler, M., Schultz, C., van Hest, J.C., van Delft, F.L., and Lemke, E.A. (2012). Genetic encoding of a bicyclo[6.1.0]nonyne-charged amino acid enables fast cellular protein imaging by metal-free ligation. ChemBioChem *13*, 2094–2099.

Bosmann, H.B., Hagopian, A., and Eylar, E.H. (1968). Cellular membranes: the isolation and characterization of the plasma and smooth membranes of HeLa cells. Arch. Biochem. Biophys. *128*, 51–69.

Bouvier, M., Heveker, N., Jockers, R., Marullo, S., and Milligan, G. (2007). BRET analysis of GPCR oligomerization: newer does not mean better. Nat. Methods *4*, 3–4, author reply 4.

Bräse, S., Gil, C., Knepper, K., and Zimmermann, V. (2005). Organic azides: an exploding diversity of a unique class of compounds. Angew. Chem. Int. Ed. Engl. *44*, 5188–5240.

Burke, J.F., and Mogg, A.E. (1985). Suppression of a nonsense mutation in mammalian cells in vivo by the aminoglycoside antibiotics G-418 and paromomycin. Nucleic Acids Res. *13*, 6265–6272.

Calebiro, D., Rieken, F., Wagner, J., Sungkaworn, T., Zabel, U., Borzi, A., Cocucci, E., Zürn, A., and Lohse, M.J. (2013). Single-molecule analysis of fluorescently labeled G-protein-coupled receptors reveals complexes with distinct dynamics and organization. Proc. Natl. Acad. Sci. USA *110*, 743–748.

Carnes, J., Jacobson, M., Leinwand, L., and Yarus, M. (2003). Stop codon suppression via inhibition of eRF1 expression. RNA *9*, 648–653.

Cassan, M., and Rousset, J.P. (2001). UAG readthrough in mammalian cells: effect of upstream and downstream stop codon contexts reveal different signals. BMC Mol. Biol. *2*, 3.

Chang, P.V., Prescher, J.A., Hangauer, M.J., and Bertozzi, C.R. (2007). Imaging cell surface glycans with bioorthogonal chemical reporters. J. Am. Chem. Soc. *129*, 8400–8401.

Chen, I., Howarth, M., Lin, W., and Ting, A.Y. (2005). Site-specific labeling of cell surface proteins with biophysical probes using biotin ligase. Nat. Methods *2*, 99–104.

Chin, J.W. (2014). Expanding and reprogramming the genetic code of cells and animals. Annu. Rev. Biochem. *83*, 379–408.

Chin, J.W., Cropp, T.A., Anderson, J.C., Mukherji, M., Zhang, Z., and Schultz, P.G. (2003). An expanded eukaryotic genetic code. Science *301*, 964–967.

Christopoulos, A., and Kenakin, T. (2002). G protein-coupled receptor allosterism and complexing. Pharmacol. Rev. *54*, 323–374.

Ciruela, F., Vilardaga, J.P., and Fernández-Dueñas, V. (2010). Lighting up multiprotein complexes: lessons from GPCR oligomerization. Trends Biotechnol. *28*, 407–415.

Cohen, A.S., Dubikovskaya, E.A., Rush, J.S., and Bertozzi, C.R. (2010). Real-time bioluminescence imaging of glycans on live cells. J. Am. Chem. Soc. *132*, 8563–8565.

Coin, I., Katritch, V., Sun, T., Xiang, Z., Siu, F.Y., Beyermann, M., Stevens, R.C., and Wang, L. (2013). Genetically encoded chemical probes in cells reveal the binding path of urocortin-I to CRF class B GPCR. Cell *155*, 1258–1269.

Conard, S.E., Buckley, J., Dang, M., Bedwell, G.J., Carter, R.L., Khass, M., and Bedwell, D.M. (2012). Identification of eRF1 residues that play critical and complementary roles in stop codon recognition. RNA *18*, 1210–1221.

Cordomí, A., Gómez-Tamayo, J.C., Gigoux, V., and Fourmy, D. (2013). Sulfur-containing amino acids in 7TMRs: molecular gears for pharmacology and function. Trends Pharmacol. Sci. *34*, 320–331.

Cornish, V.W., Hahn, K.M., and Schultz, P.G. (1996). Site-specific protein modification using a ketone handle. J. Am. Chem. Soc. *118*, 8150–8151.

Daggett, K.A., and Sakmar, T.P. (2011). Site-specific in vitro and in vivo incorporation of molecular probes to study G-protein-coupled receptors. Curr. Opin. Chem. Biol. *15*, 392–398.

de Graaf, A.J., Kooijman, M., Hennink, W.E., and Mastrobattista, E. (2009). Nonnatural amino acids for site-specific protein conjugation. Bioconjug. Chem. *20*, 1281–1295.

Debets, M.F., van Berkel, S.S., Schoffelen, S., Rutjes, F.P., van Hest, J.C., and van Delft, F.L. (2010a). Aza-dibenzocyclooctynes for fast and efficient enzyme PEGylation via copper-free (3+2) cycloaddition. Chem. Commun. (Camb.) *46*, 97–99.

Debets, M.F., van der Doelen, C.W.J., Rutjes, F.P.J.T., and van Delft, F.L. (2010b). Azide: a unique dipole for metal-free bioorthogonal ligations. ChemBioChem *11*, 1168–1184.

Debets, M.F., van Berkel, S.S., Dommerholt, J., Dirks, A.T.J., Rutjes, F.P.J.T., and van Delft, F.L. (2011). Bioconjugation with strained alkenes and alkynes. Acc. Chem. Res. *44*, 805–815.

Devigny, C., Perez-Balderas, F., Hoogeland, B., Cuboni, S., Wachtel, R., Mauch, C.P., Webb, K.J., Deussing, J.M., and Hausch, F. (2011). Biomimetic screening of class-B G protein-coupled receptors. J. Am. Chem. Soc. *133*, 8927–8933.

Dommerholt, J., Schmidt, S., Temming, R., Hendriks, L.J.A., Rutjes, F.P.J.T., van Hest, J.C.M., Lefeber, D.J., Friedl, P., and van Delft, F.L. (2010). Readily accessible bicyclononynes for bioorthogonal labeling and three-dimensional imaging of living cells. Angew. Chem. Int. Ed. Engl. *49*, 9422–9425.

Dormán, G., and Prestwich, G.D. (1994). Benzophenone photophores in biochemistry. Biochemistry *33*, 5661–5673.

Dunham, T.D., and Farrens, D.L. (1999). Conformational changes in rhodopsin. Movement of helix f detected by site-specific chemical labeling and fluorescence spectroscopy. J. Biol. Chem. *274*, 1683–1690.

Fenton, A.W. (2008). Allostery: an illustrated definition for the 'second secret of life'. Trends Biochem. Sci. *33*, 420–425.

Fleissner, M.R., Brustad, E.M., Kálai, T., Altenbach, C., Cascio, D., Peters, F.B., Hideg, K., Peuker, S., Schultz, P.G., and Hubbell, W.L. (2009). Site-directed spin labeling of a genetically encoded unnatural amino acid. Proc. Natl. Acad. Sci. USA *106*, 21637–21642.

Floquet, C., Hatin, I., Rousset, J.P., and Bidou, L. (2012). Statistical analysis of readthrough levels for nonsense mutations in mammalian cells reveals a major determinant of response to gentamicin. PLoS Genet. *8*, e1002608.

Förster, T. (1948). *Zwischenmolekulare Energiewanderung und Fluoreszenz. Annalen Der Physik *2*, 55–75.

Frolova, L., Le Goff, X., Rasmussen, H.H., Cheperegin, S., Drugeon, G., Kress, M., Arman, I., Haenni, A.L., Celis, J.E., Philippe, M., et al. (1994). A highly conserved eukaryotic protein family possessing properties of polypeptide chain release factor. Nature *372*, 701–703.

Geslain, R., and Pan, T. (2010). Functional analysis of human tRNA isodecoders. J. Mol. Biol. *396*, 821–831.

Gether, U., Lin, S., Ghanouni, P., Ballesteros, J.A., Weinstein, H., and Kobilka, B.K. (1997). Agonists induce conformational changes in transmembrane domains III and VI of the beta2 adrenoceptor. EMBO J. *16*, 6737–6747.

Ghanouni, P., Steenhuis, J.J., Farrens, D.L., and Kobilka, B.K. (2001). Agonist-induced conformational changes in the G-protein-coupling domain of the beta 2 adrenergic receptor. Proc. Natl. Acad. Sci. USA *98*, 5997–6002.

Goodman, H.M., Abelson, J., Landy, A., Brenner, S., and Smith, J.D. (1968). Amber suppression: a nucleotide change in the anticodon of a tyrosine transfer RNA. Nature *217*, 1019–1024.

Greiss, S., and Chin, J.W. (2011). Expanding the genetic code of an animal. J. Am. Chem. Soc. *133*, 14196–14199.

Grimsrud, P.A., Xie, H., Griffin, T.J., and Bernlohr, D.A. (2008). Oxidative stress and covalent modification of protein with bioactive aldehydes. J. Biol. Chem. *283*, 21837–21841.

Grunbeck, A., Huber, T., Sachdev, P., and Sakmar, T.P. (2011). Mapping the ligand-binding site on a G protein-coupled receptor (GPCR) using genetically encoded photocrosslinkers. Biochemistry *50*, 3411–3413.

Grunbeck, A., Huber, T., Abrol, R., Trzaskowski, B., Goddard, W.A., 3rd, and Sakmar, T.P. (2012). Genetically encoded photo-cross-linkers map the binding site of an allosteric drug on a G protein-coupled receptor. ACS Chem. Biol. *7*, 967–972.

Gubbens, J., Kim, S.J., Yang, Z., Johnson, A.E., and Skach, W.R. (2010). In vitro incorporation of nonnatural amino acids into protein using tRNA(Cys)-derived opal, ochre, and amber suppressor tRNAs. RNA *16*, 1660–1672.

Gurevich, V.V., Pals-Rylaarsdam, R., Benovic, J.L., Hosey, M.M., and Onorato, J.J. (1997). Agonist-receptor-arrestin, an alternative ternary complex with high agonist affinity. J. Biol. Chem. *272*, 28849–28852.

Ha, T. (2013). Single-molecule approaches embrace molecular cohorts. Cell *154*, 723–726.

Hang, H.C., and Bertozzi, C.R. (2001). Chemoselective approaches to glycoprotein assembly. Acc. Chem. Res. *34*, 727–736.

Hangauer, M.J., and Bertozzi, C.R. (2008). A FRET-based fluorogenic phosphine for live-cell imaging with the Staudinger ligation. Angew. Chem. Int. Ed. Engl. *47*, 2394–2397.

Hecht, S.M., Alford, B.L., Kuroda, Y., and Kitano, S. (1978). "Chemical aminoacylation" of tRNA's. J. Biol. Chem. *253*, 4517–4520.

Heckler, T.G., Chang, L.H., Zama, Y., Naka, T., Chorghade, M.S., and Hecht, S.M. (1984). T4 RNA ligase mediated preparation of novel "chemically misacylated" tRNAPheS. Biochemistry *23*, 1468–1473.

Hern, J.A., Baig, A.H., Mashanov, G.I., Birdsall, B., Corrie, J.E., Lazareno, S., Molloy, J.E., and Birdsall, N.J. (2010). Formation and dissociation of M1 muscarinic receptor dimers seen by total internal reflection fluorescence imaging of single molecules. Proc. Natl. Acad. Sci. USA *107*, 2693–2698.

Hinner, M.J., and Johnsson, K. (2010). How to obtain labeled proteins and what to do with them. Curr. Opin. Biotechnol. *21*, 766–776.

Hollenstein, K., de Graaf, C., Bortolato, A., Wang, M.W., Marshall, F.H., and Stevens, R.C. (2014). Insights into the structure of class B GPCRs. Trends Pharmacol. Sci. *35*, 12–22.

Horst, R., Liu, J.J., Stevens, R.C., and Wüthrich, K. (2013). β_2-adrenergic receptor activation by agonists studied with ^{19}F NMR spectroscopy. Angew. Chem. Int. Ed. Engl. *52*, 10762–10765.

Hubbell, W.L., Altenbach, C., Hubbell, C.M., and Khorana, H.G. (2003). Rhodopsin structure, dynamics, and activation: a perspective from crystallography, site-directed spin labeling, sulfhydryl reactivity, and disulfide cross-linking. Adv. Protein Chem. *63*, 243–290.

Huber, T., and Sakmar, T.P. (2008). Rhodopsin's active state is frozen like a DEER in the headlights. Proc. Natl. Acad. Sci. USA *105*, 7343–7344.

Huber, T., and Sakmar, T.P. (2011). Escaping the flatlands: new approaches for studying the dynamic assembly and activation of GPCR signaling complexes. Trends Pharmacol. Sci. *32*, 410–419.

Huber, T., Menon, S., and Sakmar, T.P. (2008). Structural basis for ligand binding and specificity in adrenergic receptors: implications for GPCR-targeted drug discovery. Biochemistry *47*, 11013–11023.

Huber, T., Naganathan, S., Tian, H., Ye, S., and Sakmar, T.P. (2013). Unnatural amino acid mutagenesis of GPCRs using amber codon suppression and bioorthogonal labeling. Methods Enzymol. *520*, 281–305.

Huisgen, R., Szeimies, G., and Mobius, L. (1967). 1.3-Dipolare Cycloadditionen 0.32. Kinetik Der Additionen Organischer Azide an CC-Mehrfachbindungen. Chemische Berichte-Recueil *100*, 2494–2507.

Ibba, M., and Soll, D. (2000). Aminoacyl-tRNA synthesis. Annu. Rev. Biochem. *69*, 617–650.

Ilegems, E., Pick, H.M., and Vogel, H. (2004). Downregulation of eRF1 by RNA interference increases mis-acylated tRNA suppression efficiency in human cells. Protein Eng. Des. Sel. *17*, 821–827.

James, J.R., Oliveira, M.I., Carmo, A.M., Iaboni, A., and Davis, S.J. (2006). A rigorous experimental framework for detecting protein oligomerization using bioluminescence resonance energy transfer. Nat. Methods *3*, 1001–1006.

Jewett, J.C., Sletten, E.M., and Bertozzi, C.R. (2010). Rapid Cu-free click chemistry with readily synthesized biarylazacyclooctynones. J. Am. Chem. Soc. *132*, 3688–3690.

Jones, D.P. (2010). Redox sensing: orthogonal control in cell cycle and apoptosis signalling. J. Intern. Med. *268*, 432–448.

Karnik, S.S., Sakmar, T.P., Chen, H.B., and Khorana, H.G. (1988). Cysteine residues 110 and 187 are essential for the formation of correct structure in bovine rhodopsin. Proc. Natl. Acad. Sci. USA *85*, 8459–8463.

Kasai, R.S., Suzuki, K.G.N., Prossnitz, E.R., Koyama-Honda, I., Nakada, C., Fujiwara, T.K., and Kusumi, A. (2011). Full characterization of GPCR monomer-dimer dynamic equilibrium by single molecule imaging. J. Cell Biol. *192*, 463–480.

Katritch, V., and Abagyan, R. (2011). GPCR agonist binding revealed by modeling and crystallography. Trends Pharmacol. Sci. *32*, 637–643.

Kenakin, T., and Christopoulos, A. (2013). Signalling bias in new drug discovery: detection, quantification and therapeutic impact. Nat. Rev. Drug Discov. *12*, 205–216.

Kent, R.S., De Lean, A., and Lefkowitz, R.J. (1980). A quantitative analysis of beta-adrenergic receptor interactions: resolution of high and low affinity states of the receptor by computer modeling of ligand binding data. Mol. Pharmacol. *17*, 14–23.

Keppler, A., Gendreizig, S., Gronemeyer, T., Pick, H., Vogel, H., and Johnsson, K. (2003). A general method for the covalent labeling of fusion proteins with small molecules in vivo. Nat. Biotechnol. *21*, 86–89.

Kiick, K.L., Saxon, E., Tirrell, D.A., and Bertozzi, C.R. (2002). Incorporation of azides into recombinant proteins for chemoselective modification by the Staudinger ligation. Proc. Natl. Acad. Sci. USA *99*, 19–24.

Klippenstein, V., Ghisi, V., Wietstruk, M., and Plested, A.J.R. (2014). Photoinactivation of glutamate receptors by genetically encoded unnatural amino acids. J. Neurosci. *34*, 980–991.

Knepp, A.M., Grunbeck, A., Banerjee, S., Sakmar, T.P., and Huber, T. (2011). Direct measurement of thermal stability of expressed CCR5 and stabilization by small molecule ligands. Biochemistry *50*, 502–511.

Kobayashi, T., Sakamoto, K., Takimura, T., Sekine, R., Kelly, V.P., Kamata, K., Nishimura, S., and Yokoyama, S. (2005). Structural basis of nonnatural amino acid recognition by an engineered aminoacyl-tRNA synthetase for genetic code expansion. Proc. Natl. Acad. Sci. USA *102*, 1366–1371.

Kobilka, B., and Schertler, G.F.X. (2008). New G-protein-coupled receptor crystal structures: insights and limitations. Trends Pharmacol. Sci. *29*, 79–83.

Köhn, M., and Breinbauer, R. (2004). The Staudinger ligation-a gift to chemical biology. Angew. Chem. Int. Ed. Engl. *43*, 3106–3116.

Kolb, H.C., Finn, M.G., and Sharpless, K.B. (2001). Click chemistry: diverse chemical function from a few good reactions. Angew. Chem. Int. Ed. Engl. *40*, 2004–2021.

Kostenis, E., and Mohr, K. (1996). Two-point kinetic experiments to quantify allosteric effects on radioligand dissociation. Trends Pharmacol. Sci. *17*, 280–283.

Kusumi, A., Shirai, Y.M., Koyama-Honda, I., Suzuki, K.G.N., and Fujiwara, T.K. (2010). Hierarchical organization of the plasma membrane: investigations by single-molecule tracking vs. fluorescence correlation spectroscopy. FEBS Lett. *584*, 1814–1823.

Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., et al.; International Human Genome Sequencing Consortium (2001). Initial sequencing and analysis of the human genome. Nature *409*, 860–921.

Lang, K., Davis, L., Torres-Kolbus, J., Chou, C., Deiters, A., and Chin, J.W. (2012). Genetically encoded norbornene directs site-specific cellular protein labelling via a rapid bioorthogonal reaction. Nat. Chem. *4*, 298–304.

Laski, F.A., Belagaje, R., RajBhandary, U.L., and Sharp, P.A. (1982). An amber suppressor tRNA gene derived by site-specific mutagenesis: cloning and function in mammalian cells. Proc. Natl. Acad. Sci. USA *79*, 5813–5817.

Laughlin, S.T., and Bertozzi, C.R. (2007). Metabolic labeling of glycans with azido sugars and subsequent glycan-profiling and visualization via Staudinger ligation. Nat. Protoc. *2*, 2930–2944.

Lavner, Y., and Kotlar, D. (2005). Codon bias as a factor in regulating expression via translation rate in the human genome. Gene *345*, 127–138.

Lazareno, S., and Birdsall, N.J.M. (1995). Detection, quantitation, and verification of allosteric interactions of agents with labeled and unlabeled ligands at G protein-coupled receptors: interactions of strychnine and acetylcholine at muscarinic receptors. Mol. Pharmacol. *48*, 362–378.

Lemieux, G.A., De Graffenried, C.L., and Bertozzi, C.R. (2003). A fluorogenic dye activated by the staudinger ligation. J. Am. Chem. Soc. *125*, 4708–4709.

Lin, W.C., Iversen, L., Tu, H.L., Rhodes, C., Christensen, S.M., Iwig, J.S., Hansen, S.D., Huang, W.Y.C., and Groves, J.T. (2014). H-Ras forms dimers on

membrane surfaces via a protein-protein interface. Proc. Natl. Acad. Sci. USA *111*, 2996–3001.

Link, A.J., Vink, M.K.S., Agard, N.J., Prescher, J.A., Bertozzi, C.R., and Tirrell, D.A. (2006). Discovery of aminoacyl-tRNA synthetase activity through cell-surface display of noncanonical amino acids. Proc. Natl. Acad. Sci. USA *103*, 10180–10185.

Liu, C.C., and Schultz, P.G. (2010). Adding new chemistries to the genetic code. Annu. Rev. Biochem. *79*, 413–444.

Liu, J.J., Horst, R., Katritch, V., Stevens, R.C., and Wüthrich, K. (2012). Biased signaling pathways in β2-adrenergic receptor characterized by 19F-NMR. Science *335*, 1106–1110.

Lohse, M.J., Nuber, S., and Hoffmann, C. (2012). Fluorescence/bioluminescence resonance energy transfer techniques to study G-protein-coupled receptor activation and signaling. Pharmacol. Rev. *64*, 299–336.

Ludeman, M.J., Kataoka, H., Srinivasan, Y., Esmon, N.L., Esmon, C.T., and Coughlin, S.R. (2005). PAR1 cleavage and signaling in response to activated protein C and thrombin. J. Biol. Chem. *280*, 13122–13128.

MacPherson, J.M., and Roy, K.L. (1986). Two human tyrosine tRNA genes contain introns. Gene *42*, 101–106.

Mahal, L.K., Yarema, K.J., and Bertozzi, C.R. (1997). Engineering chemical reactivity on cell surfaces through oligosaccharide biosynthesis. Science *276*, 1125–1128.

Malik, V., Rodino-Klapac, L.R., Viollet, L., and Mendell, J.R. (2010). Aminoglycoside-induced mutation suppression (stop codon readthrough) as a therapeutic strategy for Duchenne muscular dystrophy. Ther. Adv. Neurol. Disord. *3*, 379–389.

Martin, R., Mogg, A.E., Heywood, L.A., Nitschke, L., and Burke, J.F. (1989). Aminoglycoside suppression at UAG, UAA and UGA codons in Escherichia coli and human tissue culture cells. Mol. Gen. Genet. *217*, 411–418.

Maurel, D., Comps-Agrar, L., Brock, C., Rives, M.L., Bourrier, E., Ayoub, M.A., Bazin, H., Tinel, N., Durroux, T., Prézeau, L., et al. (2008). Cell-surface protein-protein interaction analysis with time-resolved FRET and snap-tag technologies: application to GPCR oligomerization. Nat. Methods *5*, 561–567.

Meldal, M., and Tornøe, C.W. (2008). Cu-catalyzed azide-alkyne cycloaddition. Chem. Rev. *108*, 2952–3015.

Menon, S.T., Han, M., and Sakmar, T.P. (2001). Rhodopsin: structural basis of molecular physiology. Physiol. Rev. *81*, 1659–1688.

Merchant, K.A., Best, R.B., Louis, J.M., Gopich, I.V., and Eaton, W.A. (2007). Characterizing the unfolded states of proteins using single-molecule FRET spectroscopy and molecular simulations. Proc. Natl. Acad. Sci. USA *104*, 1528–1533.

Mielke, T., Alexiev, U., Gläsel, M., Otto, H., and Heyn, M.P. (2002). Light-induced changes in the structure and accessibility of the cytoplasmic loops of rhodopsin in the activated MII state. Biochemistry *41*, 7875–7884.

Mukai, T., Wakiyama, M., Sakamoto, K., and Yokoyama, S. (2010). Genetic encoding of non-natural amino acids in Drosophila melanogaster Schneider 2 cells. Protein Sci. *19*, 440–448.

Muschielok, A., Andrecka, J., Jawhari, A., Brückner, F., Cramer, P., and Michaelis, J. (2008). A nano-positioning system for macromolecular structural analysis. Nat. Methods *5*, 965–971.

Naganathan, S., Grunbeck, A., Tian, H., Huber, T., and Sakmar, T.P. (2013a). Genetically-encoded molecular probes to study G protein-coupled receptors. J. Vis. Exp. (79), e50588.

Naganathan, S., Ye, S., Sakmar, T.P., and Huber, T. (2013b). Site-specific epitope tagging of G protein-coupled receptors by bioorthogonal modification of a genetically encoded unnatural amino acid. Biochemistry *52*, 1028–1036.

Nakamura, Y., Gojobori, T., and Ikemura, T. (2000). Codon usage tabulated from international DNA sequence databases: status for the year 2000. Nucleic Acids Res. *28*, 292.

Ning, X., Guo, J., Wolfert, M.A., and Boons, G.J. (2008). Visualizing metabolically labeled glycoconjugates of living cells by copper-free and fast huisgen cycloadditions. Angew. Chem. Int. Ed. Engl. *47*, 2253–2255.

Noren, C.J., Anthony-Cahill, S.J., Griffith, M.C., and Schultz, P.G. (1989). A general method for site-specific incorporation of unnatural amino acids into proteins. Science *244*, 182–188.

Novoa, E.M., Pavon-Eternod, M., Pan, T., and Ribas de Pouplana, L. (2012). A role for tRNA modifications in genome structure and codon usage. Cell *149*, 202–213.

Nygaard, R., Zou, Y., Dror, R.O., Mildorf, T.J., Arlow, D.H., Manglik, A., Pan, A.C., Liu, C.W., Fung, J.J., Bokoch, M.P., et al. (2013). The dynamic process of β(2)-adrenergic receptor activation. Cell *152*, 532–542.

Okayasu, T., Ikeda, M., Akimoto, K., and Sorimachi, K. (1997). The amino acid composition of mammalian and bacterial cells. Amino Acids *13*, 379–391.

Peleg, G., Ghanouni, P., Kobilka, B.K., and Zare, R.N. (2001). Single-molecule spectroscopy of the beta(2) adrenergic receptor: observation of conformational substates in a membrane protein. Proc. Natl. Acad. Sci. USA *98*, 8469–8474.

Pfleger, K.D.G., and Eidne, K.A. (2005). Monitoring the formation of dynamic G-protein-coupled receptor-protein complexes in living cells. Biochem. J. *385*, 625–637.

Phillips-Jones, M.K., Hill, L.S.J., Atkinson, J., and Martin, R. (1995). Context effects on misreading and suppression at UAG codons in human cells. Mol. Cell. Biol. *15*, 6593–6600.

Prescher, J.A., and Bertozzi, C.R. (2005). Chemistry in living systems. Nat. Chem. Biol. *1*, 13–21.

Prescher, J.A., Dube, D.H., and Bertozzi, C.R. (2004). Chemical remodelling of cell surfaces in living animals. Nature *430*, 873–877.

Prummer, M., Meyer, B.H., Franzini, R., Segura, J.M., George, N., Johnsson, K., and Vogel, H. (2006). Post-translational covalent labeling reveals heterogeneous mobility of individual G protein-coupled receptors in living cells. ChemBioChem *7*, 908–911.

Rajagopal, S., Rajagopal, K., and Lefkowitz, R.J. (2010). Teaching old receptors new tricks: biasing seven-transmembrane receptors. Nat. Rev. Drug Discov. *9*, 373–386.

Rasmussen, S.G., DeVree, B.T., Zou, Y., Kruse, A.C., Chung, K.Y., Kobilka, T.S., Thian, F.S., Chae, P.S., Pardon, E., Calinski, D., et al. (2011). Crystal structure of the β2 adrenergic receptor-Gs protein complex. Nature *477*, 549–555.

Ray-Saha, S., Huber, T., and Sakmar, T.P. (2014). Antibody epitopes on g protein-coupled receptors mapped with genetically encoded photoactivatable cross-linkers. Biochemistry *53*, 1302–1310.

Reddington, S.C., Tippmann, E.M., and Jones, D.D. (2012). Residue choice defines efficiency and influence of bioorthogonal protein modification via genetically encoded strain promoted Click chemistry. Chem. Commun. (Camb.) *48*, 8419–8421.

Resek, J.F., Farahbakhsh, Z.T., Hubbell, W.L., and Khorana, H.G. (1993). Formation of the meta II photointermediate is accompanied by conformational changes in the cytoplasmic surface of rhodopsin. Biochemistry *32*, 12025–12032.

Rostovtsev, V.V., Green, L.G., Fokin, V.V., and Sharpless, K.B. (2002). A stepwise huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. Angew. Chem. Int. Ed. Engl. *41*, 2596–2599.

Rubino, J.T., Chenkin, M.P., Keller, M., Riggs-Gelasco, P., and Franz, K.J. (2011). A comparison of methionine, histidine and cysteine in copper(I)-binding peptides reveals differences relevant to copper uptake by organisms in diverse environments. Metallomics *3*, 61–73.

Sage, D., Neumann, F.R., Hediger, F., Gasser, S.M., and Unser, M. (2005). Automatic tracking of individual fluorescence particles: application to the study of chromosome dynamics. IEEE Trans. Image Process. *14*, 1372–1383.

Sakamoto, K., Hayashi, A., Sakamoto, A., Kiga, D., Nakayama, H., Soma, A., Kobayashi, T., Kitabatake, M., Takio, K., Saito, K., et al. (2002). Site-specific incorporation of an unnatural amino acid into proteins in mammalian cells. Nucleic Acids Res. *30*, 4692–4699.

Sakmar, T.P. (2011). Receptors: clicking class B GPCR ligands. Nat. Chem. Biol. *7*, 500–501.

Salahpour, A., and Masri, B. (2007). Experimental challenge to a 'rigorous' BRET analysis of GPCR oligomerization. Nat. Methods *4*, 599–600, author reply 601.

Sarramegna, V., Talmont, F., Demange, P., and Milon, A. (2003). Heterologous expression of G-protein-coupled receptors: comparison of expression systems from the standpoint of large-scale production and purification. Cell. Mol. Life Sci. *60*, 1529–1546.

Saxon, E., and Bertozzi, C.R. (2000). Cell surface engineering by a modified Staudinger reaction. Science *287*, 2007–2010.

Saxon, E., Luchansky, S.J., Hang, H.C., Yu, C., Lee, S.C., and Bertozzi, C.R. (2002). Investigating cellular metabolism of synthetic azidosugars with the Staudinger ligation. J. Am. Chem. Soc. *124*, 14893–14902.

Schilling, C.I., Jung, N., Biskup, M., Schepers, U., and Bräse, S. (2011). Bioconjugation via azide-Staudinger ligation: an overview. Chem. Soc. Rev. *40*, 4840–4871.

Schröder, G.F., Alexiev, U., and Grubmüller, H. (2005). Simulation of fluorescence anisotropy experiments: probing protein dynamics. Biophys. J. *89*, 3757–3770.

Seitz, G., Pohl, L., and Pohlke, R. (1969). 5,6-Didehydro-11,12-dihydrodibenzo [a,E]dycloоctene. Angew. Chem. Int. Ed. *8*, 447–448.

Shi, X., Jung, Y., Lin, L.-J., Liu, C., Wu, C., Cann, I.K.O., and Ha, T. (2012). Quantitative fluorescence labeling of aldehyde-tagged proteins for single-molecule imaging. Nat. Methods *9*, 499–503.

Sindbert, S., Kalinin, S., Nguyen, H., Kienzler, A., Clima, L., Bannwarth, W., Appel, B., Müller, S., and Seidel, C.A. (2011). Accurate distance determination of nucleic acids via Förster resonance energy transfer: implications of dye linker length and rigidity. J. Am. Chem. Soc. *133*, 2463–2480.

Slavoff, S.A., Chen, I., Choi, Y.A., and Ting, A.Y. (2008). Expanding the substrate tolerance of biotin ligase through exploration of enzymes from diverse species. J. Am. Chem. Soc. *130*, 1160–1162.

Sletten, E.M., and Bertozzi, C.R. (2011). From mechanism to mouse: a tale of two bioorthogonal reactions. Acc. Chem. Res. *44*, 666–676.

Snaar-Jagalska, B.E., Cambi, A., Schmidt, T., and de Keijzer, S. (2013). Single-molecule imaging technique to study the dynamic regulation of GPCR function at the plasma membrane. Methods Enzymol. *521*, 47–67.

Sommerhage, F., Helpenstein, R., Rauf, A., Wrobel, G., Offenhäusser, A., and Ingebrandt, S. (2008). Membrane allocation profiling: a method to characterize three-dimensional cell shape and attachment based on surface reconstruction. Biomaterials *29*, 3927–3935.

Song, H., Mugnier, P., Das, A.K., Webb, H.M., Evans, D.R., Tuite, M.F., Hemmings, B.A., and Barford, D. (2000). The crystal structure of human eukaryotic release factor eRF1—mechanism of stop codon recognition and peptidyl-tRNA hydrolysis. Cell *100*, 311–321.

Srinivasan, G., James, C.M., and Krzycki, J.A. (2002). Pyrrolysine encoded by UAG in Archaea: charging of a UAG-decoding specialized tRNA. Science *296*, 1459–1462.

Stadtman, E.R. (1993). Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. Annu. Rev. Biochem. *62*, 797–821.

Stadtman, E.R., and Levine, R.L. (2003). Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. Amino Acids *25*, 207–218.

Steyaert, J., and Kobilka, B.K. (2011). Nanobody stabilization of G protein-coupled receptor conformational states. Curr. Opin. Struct. Biol. *21*, 567–572.

Stryer, L., and Haugland, R.P. (1967). Energy transfer: a spectroscopic ruler. Proc. Natl. Acad. Sci. USA *58*, 719–726.

Suzuki, K., Ritchie, K., Kajikawa, E., Fujiwara, T., and Kusumi, A. (2005). Rapid hop diffusion of a G-protein-coupled receptor in the plasma membrane as revealed by single-molecule techniques. Biophys. J. *88*, 3659–3680.

Thirumurugan, P., Matosiuk, D., and Jozwiak, K. (2013). Click chemistry for drug development and diverse chemical-biology applications. Chem. Rev. *113*, 4905–4979.

Tian, H., Sakmar, T.P., and Huber, T. (2013). Site-specific labeling of genetically encoded azido groups for multicolor, single-molecule fluorescence imaging of GPCRs. Methods Cell Biol. *117*, 267–303.

Tian, H., Naganathan, S., Kazmi, M.A., Schwartz, T.W., Sakmar, T.P., and Huber, T. (2014). Bioorthogonal fluorescent labeling of functional g-protein-coupled receptors. ChemBioChem *15*, 1820–1829.

Tinnefeld, P., and Sauer, M. (2005). Branching out of single-molecule fluorescence spectroscopy: challenges for chemistry and influence on biology. Angew. Chem. Int. Ed. Engl. *44*, 2642–2671.

Tornøe, C.W., Christensen, C., and Meldal, M. (2002). Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(i)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. J. Org. Chem. *67*, 3057–3064.

Tsao, M.-L., Tian, F., and Schultz, P.G. (2005). Selective Staudinger modification of proteins containing p-azidophenylalanine. ChemBioChem *6*, 2147–2149.

Valentin-Hansen, L., Park, M., Huber, T., Grunbeck, A., Naganathan, S., Schwartz, T.W., and Sakmar, T.P. (2014). Mapping substance p binding sites on the neurokinin-1 receptor using genetic incorporation of a photoreactive amino acid. J. Biol. Chem. *289*, 18045–18054.

van Berkel, S.S., Dirks, A.T.J., Debets, M.F., van Delft, F.L., Cornelissen, J.J.L.M., Nolte, R.J.M., and Rutjes, F.P.J.T. (2007). Metal-free triazole formation as a tool for bioconjugation. ChemBioChem *8*, 1504–1508.

van Berkel, S.S., van Eldijk, M.B., and van Hest, J.C.M. (2011). Staudinger ligation as a method for bioconjugation. Angew. Chem. Int. Ed. Engl. *50*, 8806–8827.

van Geel, R., Pruijn, G.J.M., van Delft, F.L., and Boelens, W.C. (2012). Preventing thiol-yne addition improves the specificity of strain-promoted azide-alkyne cycloaddition. Bioconjug. Chem. *23*, 392–398.

van Tol, H., Stange, N., Gross, H.J., and Beier, H. (1987). A human and a plant intron-containing tRNATyr gene are both transcribed in a HeLa cell extract but spliced along different pathways. EMBO J. *6*, 35–41.

Vilardaga, J.-P., Bünemann, M., Feinstein, T.N., Lambert, N., Nikolaev, V.O., Engelhardt, S., Lohse, M.J., and Hoffmann, C. (2009). GPCR and G proteins: drug efficacy and activation in live cells. Mol. Endocrinol. *23*, 590–599.

Vogelsang, J., Kasper, R., Steinhauer, C., Person, B., Heilemann, M., Sauer, M., and Tinnefeld, P. (2008). A reducing and oxidizing system minimizes photobleaching and blinking of fluorescent dyes. Angew. Chem. Int. Ed. Engl. *47*, 5465–5469.

Wang, L., Brock, A., Herberich, B., and Schultz, P.G. (2001). Expanding the genetic code of *Escherichia coli*. Science *292*, 498–500.

Wang, L., and Schultz, P.G. (2004). Expanding the genetic code. Angew. Chem. Int. Ed. Engl. *44*, 34–66.

Wang, L., Zhang, Z., Brock, A., and Schultz, P.G. (2003). Addition of the keto functional group to the genetic code of Escherichia coli. Proc. Natl. Acad. Sci. USA *100*, 56–61.

White, S.S., Li, H., Marsh, R.J., Piper, J.D., Leonczek, N.D., Nicolaou, N., Bain, A.J., Ying, L., and Klenerman, D. (2006). Characterization of a single molecule DNA switch in free solution. J. Am. Chem. Soc. *128*, 11423–11432.

Wittig, G., and Krebs, A. (1961). Zur existenz niedergliedriger cycloalkine 0.1. Chemische Berichte-Recueil *94*, 3260–3275.

Wong, C.H., and Zimmerman, S.C. (2013). Orthogonality in organic, polymer, and supramolecular chemistry: from Merrifield to click chemistry. Chem. Commun. (Camb.) *49*, 1679–1695.

Wu, Z., Fernandez-Lima, F.A., and Russell, D.H. (2010). Amino acid influence on copper binding to peptides: cysteine versus arginine. J. Am. Soc. Mass Spectrom. *21*, 522–533.

Yanagisawa, T., Ishii, R., Fukunaga, R., Kobayashi, T., Sakamoto, K., and Yokoyama, S. (2008). Multistep engineering of pyrrolysyl-tRNA synthetase to genetically encode N(epsilon)-(o-azidobenzyloxycarbonyl) lysine for site-specific protein modification. Chem. Biol. *15*, 1187–1197.

Yao, J.Z., Uttamapinant, C., Poloukhtine, A., Baskin, J.M., Codelli, J.A., Sletten, E.M., Bertozzi, C.R., Popik, V.V., and Ting, A.Y. (2012). Fluorophore targeting to cellular proteins via enzyme-mediated azide ligation and strain-promoted cycloaddition. J. Am. Chem. Soc. *134*, 3720–3728.

Yaremchuk, A., Kriklivyi, I., Tukalo, M., and Cusack, S. (2002). Class I tyrosyl-tRNA synthetase has a class II mode of cognate tRNA recognition. EMBO J. *21*, 3829–3840.

Ye, S., Köhrer, C., Huber, T., Kazmi, M., Sachdev, P., Yan, E.C., Bhagat, A., RajBhandary, U.L., and Sakmar, T.P. (2008). Site-specific incorporation of keto amino acids into functional G protein-coupled receptors using unnatural amino acid mutagenesis. J. Biol. Chem. *283*, 1525–1533.

Ye, S., Huber, T., Vogel, R., and Sakmar, T.P. (2009). FTIR analysis of GPCR activation using azido probes. Nat. Chem. Biol. *5*, 397–399.

Ye, S., Zaitseva, E., Caltabiano, G., Schertler, G.F.X., Sakmar, T.P., Deupi, X., and Vogel, R. (2010). Tracking G-protein-coupled receptor activation using genetically encoded infrared probes. Nature *464*, 1386–1389.

Ye, S., Riou, M., Carvalho, S., and Paoletti, P. (2013). Expanding the genetic code in Xenopus laevis oocytes. ChemBioChem *14*, 230–235.

Zhang, Z., Smith, B.A.C., Wang, L., Brock, A., Cho, C., and Schultz, P.G. (2003). A new strategy for the site-specific modification of proteins in vivo. Biochemistry *42*, 6735–6746.

Zhu, Q., and Casey, J.R. (2007). Topology of transmembrane proteins by scanning cysteine accessibility mutagenesis methodology. Methods *41*, 439–450.

Zhu, S., Riou, M., Yao, C.A., Carvalho, S., Rodriguez, P.C., Bensaude, O., Paoletti, P., and Ye, S. (2014). Genetically encoding a light switch in an ionotropic glutamate receptor reveals subunit-specific interfaces. Proc. Natl. Acad. Sci. USA *111*, 6081–6086.